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Effect of select nitrocompounds on ruminal fermentation; an initial look at their potential to reduce economic and environmental costs associated with ruminal methanogenesis

Robin C. Anderson [a,*], Todd R. Callaway [a], Jo Ann S. Van Kessel [b], Yong Soo Jung [a], Thomas S. Edrington [a], David J. Nisbet [a]

[a] United States Department of Agriculture, Agricultural Research Service, Southern Plains Agricultural Research Center, Food and Feed Safety Research Unit, 2881 F&B Rd., College Station, TX 77845, USA
[b] United States Department of Agriculture, Agricultural Research Service, Animal Waste Pathogen Laboratory, 10300 Baltimore Blvd, Beltsville, MD 20705, USA

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Abstract

Methane production by ruminal microbes during the digestion of feedstuffs is an inefficient process resulting in losses of 2–12% of the gross energy consumed by ruminants. Presently, we report the effect of three inhibitors on ruminal methane production in vitro. Mixed populations of ruminal microbes collected from cannulated cows maintained on an alfalfa hay:corn diet (50:50) were incubated at 39 °C for 24 h under a 100% carbon dioxide gas phase in closed tubes with 72 mM added sodium formate. Cultures were supplemented with 12 mM 2-nitropropanol, nitroethane or nitroethanol (experiment 1) or with 2, 12 or 24 mM nitroethane or a combination of 12 mM nitroethane and 4 mM nitroethanol (experiment 2). Control cultures containing no added nitrocompound were incubated simultaneously with treated incubations. Methane concentrations were reduced ($P < 0.05$) from those measured in control incubations (27.6 ± 2.1 and 17.7 ± 0.8 μmol/ml; mean ± SD for experiments 1 and 2, respectively) by at least 57% and as much as 94% in the nitrocompound supplemented incubations. By comparison, the widely fed methane inhibitor, monensin, typically reduces ruminal methane production by about 33%. Concentrations of volatile fatty acids and ammonia that accumulated in the nitrocompound supplemented incubations were not markedly affected compared to those produced by control cultures despite the reductions in methane produced. Hydrogen accumulated only slightly in cultures supplemented with the nitrocompounds. These results demonstrate that 2-nitropropanol, nitroethane and nitroethanol inhibit ruminal methane production. Further research is warranted to determine the mechanisms responsible for this inhibition and to see if these inhibitors can be used in practical application to reduce economic and environmental costs associated with ruminal methanogenesis.
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1. Introduction

Despite its beneficial role in maintaining low partial pressures of hydrogen within the ruminal microbial ecosystem, the production of methane is recognized as an energetically wasteful process to the ruminant in that it allows the conversion of useful substrates into compounds no longer useable by the host. This digestive inefficiency can result in losses of 2–12% of the gross energy consumed by the animal (Johnson and Johnson, 1995; McAllister et al., 1996). Additionally, methane is recognized as an important greenhouse gas and is thought to contribute an environmental cost to animal agriculture (Johnson and Johnson, 1995; Moss et al., 2000; Reay, 2001; Van Nevel and Demeyer, 1996). Ruminant microbiologists have long sought to develop strategies for reducing ruminal methanogenesis within the rumen ecosystem. Most methane inhibiting strategies; however, tend to compromise the beneficial fermentative efficiencies associated with interspecies hydrogen transfer as evidenced by reductions in certain digestive processes, microbial growth yields, a decreased

* Corresponding author. Tel.: +1-979-260-9317; fax: +1-979-260-9332.
E-mail address: anderson@ffsru.tamu.edu (R.C. Anderson).

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production of acetate and an increased production of reduced fermentation acids, notably propionate (Miller, 1995; Moss et al., 2000; Prins, 1979; Van Nevel and Demeyer, 1996). Feeding of the ionophore, monensin, to ruminants is perhaps the most successful example of the use of a methane inhibitor to improve animal performance but even in this case ruminal propionate production is increased at the expense of the overall fermentation efficiency (Chen and Wolin, 1979; Johnson and Johnson, 1995; Mbanzamihigo et al., 1996; Rumpler et al., 1986). Conversely, the benefits of feeding monensin on animal performance has recently been attributed to a protein sparing effect that is achieved via the direct inhibition of obligate amino acid-fermenting bacteria (Nagaraja, 1995; Yang and Russell, 1993).

Alternatively, the addition of nitrate to populations of rumen microbes enriched with *Denitrobacterium detoxificans*, a hydrogen-oxidizing, nitrate-reducing bacterium, has been reported to decrease ruminal methane production in vitro by 18-fold (Anderson and Rasmussen, 1998). In this case, reducing equivalents were used to reduce nitrate rather than carbon dioxide, with ammonia as the final product, which thus effectively preserved the hydrogen sink associated with interspecies hydrogen transfer. Presently; however, the practical use of nitrate as an alternative electron acceptor is precluded by the toxicity associated with its reduced intermediate, nitrite (McAllister et al., 1996; Pfister, 1988). In contrast, nitroethane and 2-nitropropanol are relatively innocuous nitroalkanes that are also used as electron acceptors by *D. detoxificans*, and these have been safely administered to cattle (Majak, 1992; Majak and Clark, 1980; Majak et al., 1986). Presently, we report results from studies evaluating the effect of these nitrocompounds and a related compound, nitroethanol, on methane production by ruminal bacteria in vitro. Portions of this work have been presented previously in preliminary form (Anderson et al., 2001).

2. Methods

2.1. Source of microorganisms and incubation conditions

Ruminal fluid containing mixed populations of microbes was used in all incubations and was collected from cannulated Holstein–Friesian cows maintained on a 50% alfalfa hay, 50% flaked corn diet. The ruminal fluid was incubated undiluted at 39 °C for 24 h in closed 16×150 mm crimp top tubes (10 ml/tube) except for supplementation with 72 mM sodium formate (to provide excess reductant) the nitrocompounds, or addition of *D. detoxificans* (see below). The nitrocompounds were added as small volumes (<5% vol/vol) from concentrated stock solutions to achieve 0 time concentrations as indicated. The initial head space gas phase was 100% carbon dioxide in all incubations. In experiment 2, parallel incubations were carried out with the addition of 4.4×10^6 cells of *D. detoxificans*, a ruminal bacterium capable of respiring anaerobically via coupling the oxidation of hydrogen to the reduction of nitrocompounds (Anderson et al., 2000; Anderson and Rasmussen, 1998). The *D. detoxificans* inoculum was obtained from a culture grown overnight in medium B supplemented with nitrate as the electron acceptor and was enumerated via a most probable number procedure (Anderson et al., 1996). Within each experiment, control cultures were incubated similarly with no added nitrocompound and all control and test incubations were performed in triplicate.

2.2. Analytical

Final concentrations of hydrogen and methane gas present in the headspace of mixed ruminal fluid incubations at the end of the incubation period were determined via gas chromatography of samples collected from the headspace (Allison et al., 1992; Anderson and Rasmussen, 1998). Final concentrations of acetate, propionate and butyrate from fluid samples also collected at the end of the incubation period were determined via gas chromatography (Hinton et al., 1990) and concentrations of nitrocompounds were determined colorimetrically (Majak et al., 1986).

2.3. Statistics

Tests for significance were accomplished using an analysis of variance procedure of Statistix for Windows (Tallahassee, FL); means were compared using a Tukey's multiple range test. Ruminal fluid used in the experiments was collected on separate days and since considerable variability in fermentation potential would not be unexpected in these different collections, our experiments were designed such that statistical comparisons were made within experiment only. Because no effect of *D. detoxificans* was observed for any of the variables measured in experiment 2, we present results from analysis of main treatment (nitrocompound) comparisons only.

3. Results and discussion

3.1. Effects on methane and hydrogen production

Mean amounts of methane produced by the mixed populations of ruminal microbes within control incubations (those containing no added nitrocompound) varied considerably between experiments (Tables 1 and 2). Methane production was markedly reduced ($P < 0.05$) by additions of nitrocompounds to the ruminal

Table 1
Effect of select nitrocompounds on accumulation of fermentation products during 24 h ruminal fluid incubations (experiment 1)

Treatment	Concentration of fermentation product[a] (μmol/ml ruminal fluid)							
	Acetate	Propionate	Butyrate	Total	Acetate:propionate	Ammonia	Hydrogen	Methane
None (control)	62.1 (70%)[f]	15.9 (18%)[h]	11.0 (12%)[h]	89.0	3.9[c]	76.0	0.1[e]	27.6[c]
12 mM 2-nitropropanol	47.6 (65%)[h]	14.9 (20%)[f]	10.3 (14%)[g]	72.8	3.2[e]	84.8	2.8[c]	2.6[d]
12 mM nitroethane	49.2 (68%)[g]	13.6 (19%)[g]	9.7 (13%)[g,h]	72.5	3.6[d]	83.7	1.9[d]	1.4[d]
12 mM nitroethanol	58.8 (65%)[h]	17.6 (19%)[g]	14.3 (16%)[j]	90.7	3.3[e]	74.5	2.1[c,d]	1.5[d]
Critical value[b]	24.2	6.9	5.8	36.8	0.2	31.2	0.8	2.9
SEM	5.35	1.52	1.29	8.12	0.04	6.89	0.18	0.64

[a] Values are the mean from $n = 3$. Values in parenthesis represent molar proportion as a percent of total. Total = sum of acetate + propionate + butyrate.
[b] Critical value for comparison at $P < 0.05$ from a Tukey's comparison of means (Statistix for Windows, Tallahassee, FL, USA).
[c,d,e]Means within a column containing unlike superscripts differ ($P < 0.05$).
[f,g,h]Molar proportions within a column containing unlike superscripts differ ($P < 0.05$).

Table 2
Effect of select nitrocompounds on accumulation of fermentation products during 24 h ruminal fluid incubations (experiment 2)

Treatment	Concentration of fermentation product[a] (μmol/ml ruminal fluid)							
	Acetate	Propionate	Butyrate	Total	Acetate:propionate	Ammonia	Hydrogen	Methane
None (control)	49.2[d,e] (77%)	8.5[d] (13%)	6.3[c,d] (10%)[f]	64.0[d,e]	5.8	21.1[d]	0.1[d]	17.6[c]
2 mM nitroethane	42.9[e] (75%)	8.0[d] (14%)	6.1[c,d] (11%)[f]	57.0[e]	5.8	27.7[c,d]	1.5[c]	7.4[d]
12 mM nitroethane	56.5[c,d] (78%)	9.3[d] (13%)	6.6[c,d] (9%)[f]	72.4[c,d]	6.1	35.4[c]	1.8[c]	1.1[e]
24 mM nitroethane	63.6[c] (79%)	11.5[c] (14%)	5.8[d] (7%)[g]	80.9[c]	5.7	35.2[c]	0.4[d]	0.9[e]
12 mM nitroethane plus 4 mM nitroethanol	60.7[c] (78%)	10.0[c,d] (13%)	7.4[c] (9%)[f]	78.1[c]	6.1	32.3[c]	1.5[c]	1.1[e]
Critical value[b]	8.7	2.1	1.4	10.4	1.0	9.8	0.4	4.5
SEM	2.05	0.49	0.34	2.45	0.25	2.32	0.10	1.04

[a] Values are the mean from $n = 3$. Values in parenthesis represent molar proportion as a percent of total. Total = sum of acetate + propionate + butyrate.
[b] Critical value for comparison at $P < 0.05$ from a Tukey's comparison of means (Statistix for Windows, Tallahassee, FL, USA).
[c,d,e]Means within same columns with unlike superscripts differ ($P < 0.05$).
[f,g]Molar proportions within column with unlike superscripts differ ($P < 0.05$).

fluid incubations (Table 1) and maximal inhibition with nitroethane was achieved at concentrations of 12 mM (Table 2). Hydrogen accumulated slightly within the incubations supplemented with nitrocompounds (Tables 1 and 2), suggesting that microbial interspecies-hydrogen transfer, i.e., the consumption of reducing equivalents generated during fermentation (Miller, 1995; Prins, 1979; Van Nevel and Demeyer, 1996), had not been completely optimized. In an earlier report, we demonstrated that an alternative mechanism for disposal of hydrogen was successfully maintained when mixed cultures of rumen microbes were coincubated with nitrate and nitrate-respiring *D. detoxificans* (Anderson and Rasmussen, 1998). In that report, maintenance of the electron sink by *D. detoxificans* was dependent on the presence of nitrate, but not 3-nitropropionate, both which can be used as a terminal electron acceptor by that bacterium (Anderson et al., 2000). It was concluded that 3-nitropropionate inhibited methane production via a direct, yet undefined effect against the methanogens which thus precluded its ability to competitively consume reductant (Anderson and Rasmussen, 1998). In support of this conclusion, incubation of the nitroethane supplemented cultures with added *D. detoxificans* (experiment 2) had no effect ($P > 0.05$) on the accumulation of hydrogen which thus indicates that the nitroethane effect on methanogenesis was also independent of *D. detoxificans* (values not shown). Similarly, despite their known potential to be enzymatically reduced by *D. detoxificans* (Anderson et al., 2000), the nitrocompounds did not serve as an alternative electron sink in these incubations since little if any disappearance of the nitrocompounds was observed (data not shown). Others have also reported the potential use of competitive hydrogen consuming reactions to inhibit methanogenesis, with the greatest potential for reductions in methane production achieved with the use of nitrate as an alternative electron acceptor (Allison and MacFarlane, 1988) and limited amounts of success achieved with sulfite and fumarate (Asanuma et al., 1999; Hegarty, 1999; Strocchi et al., 1994). Attempts to use acetogenic bacteria to outcompete methanogens for reducing equivalents has also been reported but again with limited success (Lopez et al., 1999).

3.2. Effects on volatile fatty acid and ammonia production

Methane inhibitors such as organic halides (Czerkawski and Breckenridge, 1975; Martin and Macy, 1985; Van Nevel et al., 1969), sodium sulfite (Van Nevel et al., 1970), 9,10-anthraquinone (Garcia-Lopez et al., 1996), and the widely used feed additive, monensin (Russell and Strobel, 1989; Slyter, 1979; Stanier and Davies, 1981) have long been known to cause notable increases in the amount of propionate produced during fermentation and this is frequently accompanied by decreased acetate production. Whereas monensin typically suppresses methane production by about 30–40% (Van Nevel and Demeyer, 1996), methane production in the present studies was reduced in a dose dependent manner with reductions as high as 94% achieved within the nitrocompound supplemented incubations (Tables 1 and 2). Consequently, we had expected to see an effect of the nitrocompounds on proportions of the reduced acids propionate and butyrate produced during the incubations. Rather, molar proportions of the reduced volatile fatty acids propionate and butyrate, were only slightly increased, if at all, in the nitrocompound supplemented incubations (Tables 1 and 2) thus indicating that reducing equivalents generated during the fermentations were not necessarily directed toward increased production of these more reduced acids. It is possible; however, that maximum accumulations of hydrogen in our incubations (2.4 kPa or 0.24 kPa/ml of ruminal fluid) never reached high enough partial pressures to promote propionate production, which according to Schulman and Valentino (1975) is maximized at hydrogen pressures of 0.188 atm (equivalent to 19 kPa). Inhibition of microbial hydrogenases generally occurs at hydrogen pressures of 1 kPa or higher (Miller, 1995); however, and it is thus possible that the reoxidation of reduced nucleotides via hydrogenase activity may have been inhibited. In the rumen, hydrogen pressure is usually 0.1 kPa, which corresponds to a concentration of approximately 1 μM (Thauer et al., 1977).

Total amounts of volatile fatty acids produced in our incubations were never significantly lower, but were on occasion higher, within the nitrocompound supplemented cultures (Tables 1 and 2). Whereas an effect ($P < 0.05$) of nitrocompound supplementation on the ratio of acetate to propionate was observed in experiment 1, this was, except for nitroethanol, due mainly to lower amounts of acetate produced (Table 1). A nitrocompound effect on the acetate:propionate ratio was not observed in experiment 2. In both experiments, ammonia accumulation was higher in the nitrocompound supplemented incubations than in controls, but significance was achieved in experiment 2 only (Tables 1 and 2). In contrast, monensin is usually, but not always, associated with a decreased amount of ammonia accumulation (Garcia-Lopez et al., 1996; Nagaraja, 1995; Russell and Martin, 1984). These results indicate that methane inhibition caused by the nitrocompounds had little if any negative effect on volatile fatty acid and ammonia production.

4. Conclusions

Evidence reported herein demonstrates that methane production by mixed populations of ruminal microbes was inhibited by 2-nitropropanol, nitroethane and nitroethanol but the mechanism responsible has yet to be determined. Our findings that total amounts and molar proportions of volatile fatty acids produced were not adversely compromised by the nitrocompounds suggests a potential role for the nitrocompounds to reduce economic and environmental costs associated with ruminal methane production. Additional research is needed, however, to determine if the beneficial effects can be realized in vivo and to further assess the long-term effects of the nitrocompounds on methane production and rumen function, especially since the rumen microbial ecosystem has shown an ability to adapt to inhibitors of methane production in the past.

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